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Conservation of capa peptide-induced nitric oxide signalling in Diptera

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Summary

In *D. melanogaster* Malpighian (renal) tubules, the capa peptides stimulate production of nitric oxide (NO) and guanosine 3′, 5′-cyclic monophosphate (cGMP), resulting in increased fluid transport. The roles of NO synthase (NOS), NO and cGMP in capa peptide signalling were tested in several other insect species of medical relevance within the Diptera (*Aedes aegypti*, *Anopheles stephensi* and *Glossina morsitans*) and in one orthopteran outgroup, *Schistocerca gregaria*. NOS immunoreactivity was detectable by immunocytochemistry in tubules from all species studied. *D. melanogaster*, *A. aegypti* and *A. stephensi* express NOS in only principal cells, whereas *G. morsitans* and *S. gregaria* show more general NOS expression in the tubule. Measurement of associated NOS activity (NADPH diaphorase) shows that both *D. melanogaster* capa-1 and the two capa peptides encoded in the *A. gambiae* genome, QGLVPFPRVamide (*Ang*CAPA-QGL) and GPTVGLFAFPRVamide (*Ang*CAPA-GPT), all stimulate NOS activity in *D. melanogaster*, *A. aegypti*, *A. stephensi* and *G. morsitans* tubules but not in *S. gregaria*. Furthermore, capa-stimulated NOS activity in all the Diptera was inhibited by the NOS inhibitor L-NAME. All capa peptides stimulate an increase in cGMP content across the dipteran species, but not in the orthopteran *S. gregaria*. Similarly, all capa peptides tested stimulate fluid secretion in *D. melanogaster*, *A. aegypti*, *A. stephensi* and *G. morsitans* tubules but are either without effect or are inhibitory on *S. gregaria*. Consistent with these results, the *Drosophila* capa receptor was shown to be expressed in *Drosophila* tubules, and its closest *Anopheles* homologue was shown to be expressed in *Anopheles* tubules. Thus, we provide the first demonstration of physiological roles for two putative *A. gambiae* neuropeptides. We also demonstrate neuropeptide modulation of fluid secretion in tsetse tubule for the first time. Finally, we show the generality of capa peptide action, to stimulate NO/cGMP signalling and increase fluid transport, across the Diptera, but not in the more primitive Orthoptera.

Key words: Malpighian tubule, fluid transport, mosquito, tsetse, capa receptor, NOS/cGMP.

Introduction

The insect renal system is composed of Malpighian tubules that vary in number and structure in different species. In all insects, however, its osmoregulatory and homeostatic functions are thought to be critical to life. Neuropeptide control of secretion by Malpighian tubules has been studied in many insect species including *Drosophila melanogaster* (Dow and Davies, 2003), *Musca domestica* (Coast, 2001a), *Rhodnius prolixus* (O'Donnell and Spring, 2000; Te Brugge et al., 2002), *Periplaneta americana* (Kay et al., 1992), *Tenebrio molitor* (Wiehart et al., 2002), *Locusta migratoria*, *Schistocerca gregaria* (Schoofs et al., 1997), *Acheta domesticus* (Spring and Clark, 1990) and *Formica polyctena* (Laenen et al., 1999, 2001). Diuresis in the disease vector species has been most studied in *Aedes aegypti* (Beyenbach, 2003; Pullikuth et al., 2003); however, less is known about tubule function in the malaria mosquito, *Anopheles gambiae*, or the tsetse fly, *Glossina morsitans*.

Drosophila melanogaster Malpighian tubules are now accepted as a genetic model of transporting epithelia (Dow and Davies, 2003). In the development of this renal model, different techniques have been developed to assess tubule function: fluid transport rates (Dow et al., 1994a), electrophysiological responses (Davies et al., 1995), ion transport (Dow, 1999) and calcium signalling using aequorin transgenes (Rosay et al., 1997). This battery of physiological assays, in combination with the powerful genetic tools associated with *Drosophila*, has allowed rapid, organotypic analysis of the cell-specific control of tubule function (Dow and Davies, 2003). Given the conserved and critical role of the tubule in insect life, findings from the *Drosophila* tubule may usefully be applied to those insect species with less developed

genomic resources but greater economic or medical significance (Dow and Davies, 2003). In particular, findings from *Drosophila* might be useful in studies of other Diptera; for example, *Aedes*, *Anopheles* and *Glossina*.

Diuresis in *Drosophila* tubules has been shown to be directly stimulated by exogenous guanosine 3′, 5′-cyclic monophosphate (cGMP), which enters tubule cells *via* a cyclic nucleotide transporter (Riegel et al., 1998), and by nitric oxide (NO; Dow et al., 1994b). NO/cGMP signalling is compartmentalised to principal cells in the main, fluid-secreting segment of tubules, containing the electrogenic vacuolar H^+-ATPase (V-ATPase) pump (Dow, 1999), which energises fluid transport. Furthermore, electrophysiological studies suggest that cGMP signalling modulates V-ATPase activity (Davies et al., 1995), suggesting that cGMP signalling may regulate ion transport in tubules.

NO/cGMP signalling is also activated by a nitridergic family of neuropeptides, capa, which comprise the only known insect NO/cGMP-mobilising peptides. Capa-1 and capa-2 are encoded by the *capa* gene in *Drosophila* (Kean et al., 2002). Both capa-1 and capa-2, as well as the closely related *Manduca sexta* CAP_{2b}, induce diuresis and stimulate NO/cGMP signalling and intracellular calcium increases in *Drosophila* tubule principal cells (Davies et al., 1995, 1997; Kean et al., 2002; Rosay et al., 1997). To date, secretion by *Aedes* and *Anopheles* tubules has not been shown to be stimulated by NO or cGMP, although the *A. stephensi* gene encoding nitric oxide synthase (NOS) has been cloned (Luckhart et al., 1998); also, *A. gambiae* tubules have been shown to express NOS transcripts (Dimopoulos et al., 1998). All known insect NOS-encoding genes are very similar (Davies, 2000), resulting in virtually identical sequences for NOS protein; as such, conservation of function at the physiological level may be anticipated. Recently, data mining of the *A. gambiae* genome has identified capa peptides in this species (Riehle et al., 2002). Although capa-like signalling beyond the Diptera can be inferred from the existence of the cardinal CAP_{2b} in the Lepidoptera, other reports have suggested that cGMP is antidiuretic in other insect orders, for example Hemiptera (Quinlan et al., 1997) and Coleoptera (Eigenheer et al., 2002, 2003), or that CAP_{2b} is without effect in Orthoptera (Coast, 2001b). It is thus of great interest to assess the phylogenetic scope of the highly unusual autocrine capa/NOS/NO/cGMP signalling model beyond *Drosophila*. Furthermore, the application of knowledge of tubule function in *D. melanogaster* to those of insect disease vectors will advance understanding of tubule physiology in the context of specific cell types and tubule regions in these animals.

Our results show that, whereas all insect tubules so far studied contain NOS (and thus have the machinery to respond to capa), only the dipteran species studied show functional responses. The scope of action of this peptide may thus be general within, but limited to, certain endopterygote orders.

Materials and methods

Insects

Drosophila melanogaster

Wild-type Oregon R flies (OrR) flies were maintained on standard *Drosophila* diet over a 12 h:12 h photoperiod at 55% humidity at 22°C.

Aedes aegypti

These were obtained as non-infective, sugar-water-fed adults from a colony maintained by Professor E. Devaney, University of Glasgow. Female animals were used upon receipt.

Anopheles stephensi *and* Anopheles gambiae

Non-infective, sugar-water-fed, adults were provided as a kind gift of Dr L. Ranford-Cartwright, University of Glasgow. Female animals were used upon receipt. If mosquitoes were not used immediately, they were maintained over a 12 h:12 h photoperiod at 55% humidity at 22°C, on 5% sucrose (v/v) solution *ad libitum* for a maximum of 3 days before use in experiments.

Glossina morsitans

Non-infective adults were provided by Dr S. Welburn, University of Edinburgh, and by Professor D. Barry, University of Glasgow. Animals were used immediately upon receipt.

Schistocerca gregaria

These were obtained from Bugs Direct (Well Cottages, Devon, UK) and either used immediately or maintained on grass over a 12 h:12 h photoperiod at 55% humidity at 22°C for a maximum of 3–4 days. All insects were cold-anaesthetised and decapitated prior to dissection to isolate intact tubules.

Peptides

Capa neuropeptides used in this study are shown in Table 1. Of the *Drosophila* capas, capa-1 (GANMGLYAFPRVamide) was used here, because of its identical mode of action to, but slightly greater potency than, capa-2 (Kean et al., 2002). Both *A. gambiae* capa peptides were synthesised: QGLVPFPRVamide (*Ang*CAPA-QGL) and GPTVGLFAFPRVamide (*Ang*CAPA-GPT). All peptides were synthesised by Invitrogen Corp. (Renfrew, UK). *A. gambiae* capa peptides were identified by data mining the *A. gambiae* genome. While this study was in progress, identical sequences for *Anopheles* capa peptides were published elsewhere (Riehle et al., 2002).

Reverse-transcription (RT)-PCR for capa receptor

Analysis of capa receptor expression in dipteran tubules was carried out by RT-PCR according to standard protocols (Dow et al., 1994b) from cDNA templates prepared from *Drosophila melanogaster*, *Anopheles stephensi* and *Anopheles gambiae* tubules. The capa receptor has been identified in

Table 1. *Members of the capa peptide family*

Origin and peptide name	Amino acid sequence	Reference
Drosophila melanogaster (CAPA1)	GANMG**L**Y**AFPRV**amide	Kean et al. (2002)
Drosophila melanogaster (CAPA2)	ASG**L**V**AFPRV**amide	Kean et al. (2002)
Anopheles gambiae (*Ang*CAPA-QGL)	QG**L**VP**FPRV**amide	Riehle et al. (2002); present study
Anopheles gambiae (*Ang*CAPA-GPT)	GPTVG**L**F**AFPRV**amide	Riehle et al. (2002); present study
Manduca sexta (CAP_{2b})	PyroE**L**Y**AFPRV**amide	Huesmann et al. (1995)

Sequences for known members of the capa family of neurohormones are shown. For clarity, common residues are highlighted in bold.

Table 2. *The epitope against which the uNOS antibody is directed is well conserved in invertebrates*

Sequence source	Order	Sequence
uNOS epitope		DQKRYHEDIFG
Drosophila melanogaster	Diptera	DESRYHEDIFG
Anopheles gambiae	Diptera	DENRYHEDIFG
Anopheles stephensi	Diptera	DENRYHEDIFG
Manduca sexta	Lepidoptera	DENRYHEDIFG
Bombyx mori	Lepidoptera	DENRYHEDIFG
Rhodnius prolixus	Homoptera	DENRYHEDIFG
Gecarcinus lateralis (blackback land crab)	Decapoda	DENRYHEDIFG

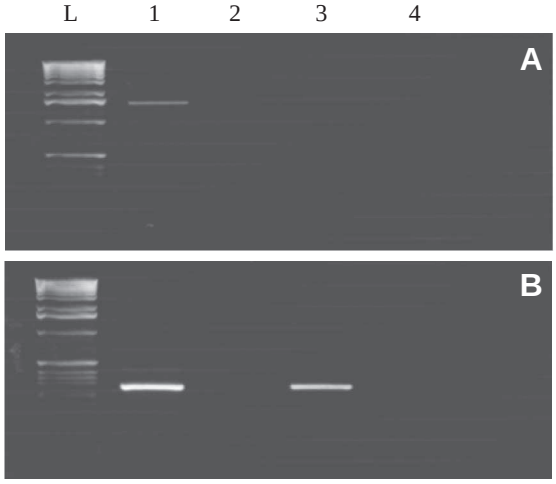

Fig. 1. Expression of capa receptor in dipteran tubules. RT-PCR of tubule cDNA templates with primers designed to the capa receptor gene from *D. melanogaster* (A, lane 1; product size, 1448 bp), *A. aegypti* (A, lane 3), *A. stephensi* (B, lane 1; product size, 250 bp), *A. gambiae* (B, lane 3; product size, 250 bp). Controls were performed using no cDNA template: *D. melanogaster* (A, lane 2), *A. aegypti* (A, lane 4), *A. stephensi* (B, lane 2), *A. gambiae* (B, lane 4). L=1 kb ladder. No PCR product was observed with *A. aegypti* cDNA template. In all cases, products obtained were of the sizes predicted for cDNA templates. Identical PCR products were obtained from both *A. gambiae* and *A. stephensi*. Very few *A. gambiae* were available for study; however, given the documented expression of the putative capa receptor in both species, the more abundant *A. stephensi* was used for all subsequent experiments.

D. melanogaster; searching the *A. gambiae* genome reveals a possible candidate for the *Anopheles* capa receptor.

For each cDNA preparation, 20 tubules were dissected, poly(A)$^+$ RNA extracted (Dynal mRNA direct kit; Dynal Biotech UK, Wirral, UK) and reverse transcribed with Superscript Plus (Gibco BRL, Invitrogen Ltd, Paisley, Renfrewshire, UK). 1 μl of the reverse transcription reaction was used as a template for PCR, containing the following gene-specific primer pairs: *Drosophila capaR* Forward, 5′-GCG-GCCGCCTAAAATGAATTCATCGACCG-3′; *Drosophila capaR* Reverse, 5′-GTCTAGAGCCTCGTGCTTAAATA-CAAG-3′; putative *A. gambiae capaR* Forward, 5′-TGTTGACCGTGTTGAAGTGTTGC-3′; putative *A. gambiae capaR* Reverse, 5′-CTGTTCTTTGCCTTTCCAATGCTC-3′. Additionally, to control against genomic contamination in cDNA preps, primers that had been designed around intron/exon boundaries of the capa receptor gene were used. Use of such primers verified the cDNA quality used in PCR reactions. Further controls were performed that included non-reverse transcribed template (i.e. no cDNA).

PCR cycle conditions for reactions with *Drosophila* cDNA template were as follows: 93°C (3 min), 36 cycles of [93°C (30 s), 54.3°C (30 s), 72°C (1 min)] and 72°C (1 min). Conditions were similar for *A. gambiae* and *A. stephensi* cDNA templates except that the annealing temperature used was 59°C. PCR products obtained from such RT-PCR experiments were cloned using the Invitrogen Topoisomerase (TOPO TA Cloning) system (Renfrew, Scotland). Cloned plasmids were purified using Qiagen kits (Crawley, UK) and sequenced to confirm their identity.

Very few *A. gambiae* were available for study; thus, for all following experiments, *A. stephensi* was used.

Immunocytochemistry

Immunocytochemistry to fixed, intact tubules from all insect species was performed using a universal anti-NOS (anti-uNOS) antibody according to previously published protocols (MacPherson et al., 2001), as described in the legend to Fig. 2. The anti-uNOS antibody is an affinity-purified rabbit universal

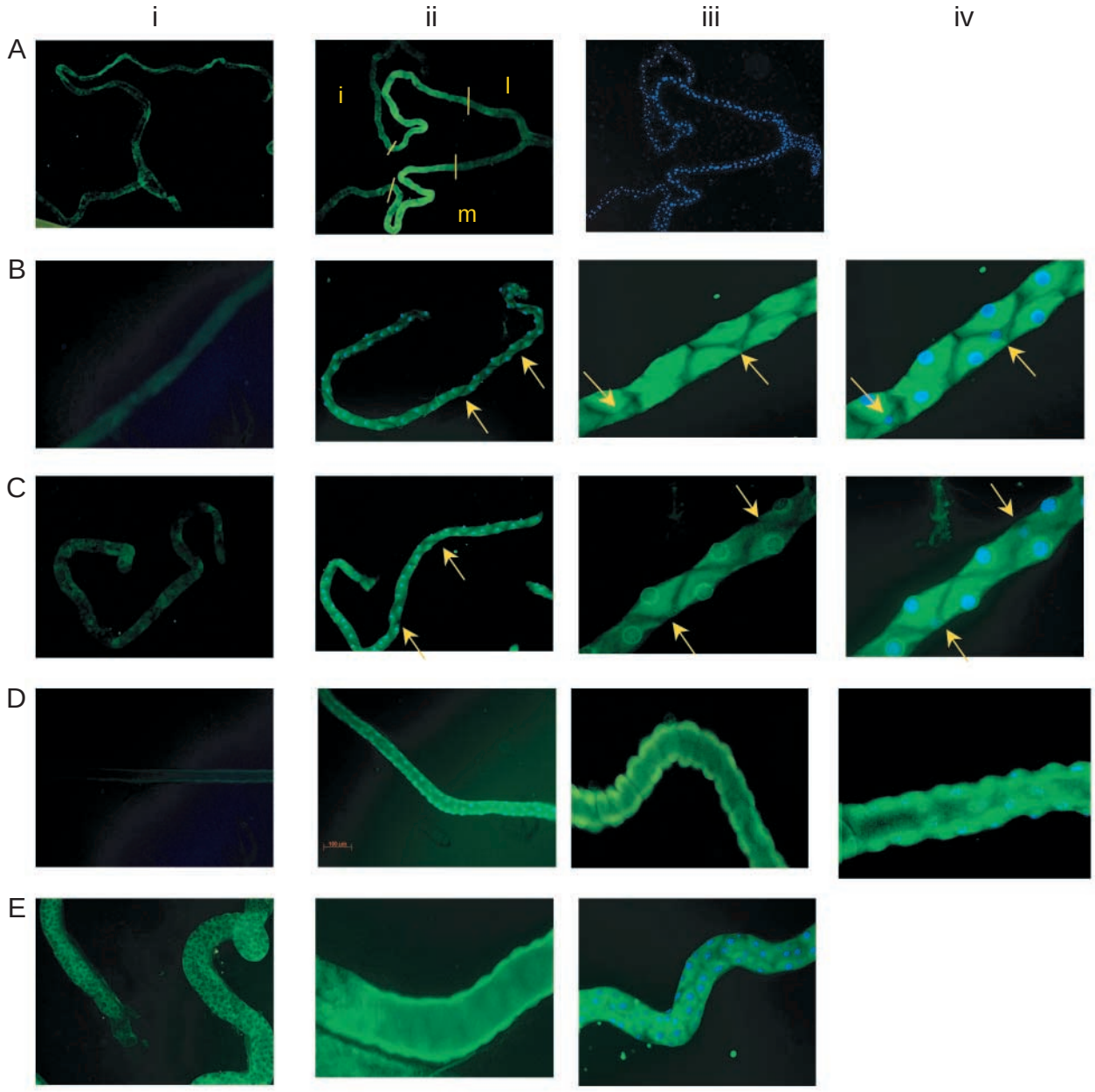

Fig. 2. Expression of nitric oxide synthase (NOS) in insect tubules. Tubules were dissected from the following species: *Drosophila melanogaster* (A); *Aedes aegypti* (B); *Anopheles stephensi* (C); *Glossina morsitans* (D) and *Schistocerca gregaria* (E). NOS distribution in intact tubules is shown using anti-uNOS antibody (Broderick et al., 2003; Dow and Davies, 2001; Gibbs and Truman, 1998); cell nuclei were visualised with DAPI (Broderick et al., 2004). Single tubules are shown in each panel, viewed by epifluorescence. Samples were viewed at 10× magnification unless stated otherwise. Tubule diameters can be taken as 35 µm. (Ai) control *Drosophila* tubule (no antibody); (Aii) pair of tubules showing NOS staining in tubule main segment (m); no staining in initial (i) and lower (l) regions; (Aiii) DAPI staining reveals cell nuclei; (Bi) control *A. aegypti* tubules (no antibody); (Bii) NOS staining throughout *A. aegypti* tubule principal cells (excluded stellate cells indicated by yellow arrow); DAPI staining reveals cell nuclei; (Biii) high magnification (50×) showing NOS staining in principal cells; unstained stellate cells indicated by yellow arrows; (Biv) NOS and DAPI-stained tubule, viewed at high magnification (50×); existence of unstained stellate cells confirmed by presence of smaller nuclei compared with principal cells, indicated by arrows; (Ci) control *A. stephensi* tubules (no antibody); (Cii) NOS staining throughout *A. stephensi* tubule principal cells (excluded stellate cells indicated by yellow arrow); DAPI staining reveals cell nuclei; (Ciii) high magnification (50×) showing NOS staining in principal cells; unstained stellate cells indicated by yellow arrows; (Civ) NOS and DAPI-stained tubule, viewed at high magnification (50×); existence of unstained stellate cells confirmed by presence of smaller nuclei compared with principal cells, indicated by arrows; (Di) control *G. morsitans* tubules (no antibody); (Dii) anti-NOS antibody-stained intact *G. morsitans* tubule at low magnification; DAPI staining reveals cell nuclei; (Diii) anti-NOS antibody-stained tubule at high magnification (20×); (Div) same preparation as Dii, viewed at high magnification (20×); (Ei) control *S. gregaria* tubules (no antibody) viewed at 20× magnification; (Eii) anti-NOS antibody-stained intact tubule viewed at 20× magnification; close-up view indicates clear staining at the membrane and cytosol; (Eiii) anti-NOS antibody-stained intact tubule viewed at 20× magnification; DAPI staining reveals cell nuclei.

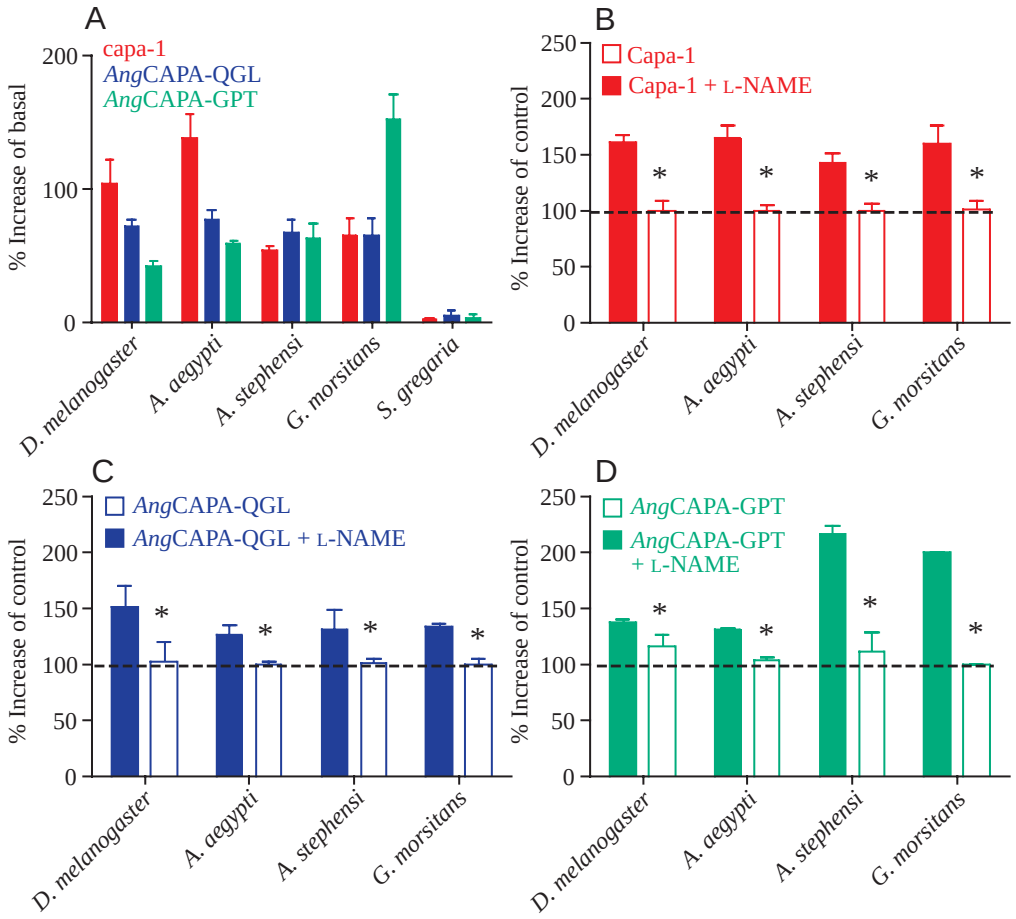

Fig. 3. NADPH diaphorase activity is stimulated by capa peptides. NADPH diaphorase activity was measured in either unstimulated or peptide-stimulated tubules from the insects shown in the absence and presence of the substrate, NADPH (A), as described in Materials and methods. Assessment of NOS-derived NADPH activity was carried out by the inclusion of a nitric oxide synthase (NOS) inhibitor in the assays (B–D). (A) Tubules were stimulated for 10 min by either capa-1 (red), *Ang*CAPA-QGL (blue) or *Ang*CAPA-GPT (green) at a final concentration of 10^{-7} mol l^{-1}. In order to normalise data for all species, results are expressed as % increase over unstimulated tubules (± S.E.M.; N=6), as described in Materials and methods. (B–D) NADPH diaphorase activity has already been shown to be an accurate estimation of NOS activity in *Drosophila melanogaster* tubules by use of an inducible transgene for NOS (Broderick et al., 2003). However, in order to investigate a direct correlation of NADPH diaphorase with NOS activity in tubules from the dipteran insects studies here, NADPH diaphorase experiments were performed in the presence of the NOS inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME). This was achieved using tubules from those insect species that showed an increase in NADPH diaphorase activity in A, as follows: (B) tubules from *Drosophila melanogaster*, *Aedes aegypti*, *Anopheles stephensi* and *Glossina morsitans* were stimulated with capa-1 in the presence of NADPH as above, in the absence (filled bars) or presence (open bars) of L-NAME, and NADPH diaphorase activity was measured. Results are expressed as % increase over control (unstimulated) tubules (± S.E.M.; N=3–8), where control values are 100%. (C) Tubules from the four dipteran species, as above, were stimulated with *Ang*CAPA-QGL in the presence of NADPH as above, in the absence (filled bars) or presence (open bars) of L-NAME, and NADPH diaphorase activity was measured. Results are expressed as % increase over control (unstimulated) tubules (± S.E.M.; N=3–8), where control values are 100%. (D) Tubules from the four dipteran species, as above, were stimulated with *Ang*CAPA-GPT in the presence of NADPH as above, in the absence (filled bars) or presence (open bars) of L-NAME, and NADPH diaphorase activity was measured. Results are expressed as % increase over control (unstimulated) tubules (± S.E.M.; N=3–8), where control values are 100%. *Statistically significant data compared with tubules in the absence of L-NAME, where P<0.05 (Student's t-test, unpaired samples).

anti-NOS antibody, used at 1:100 dilution and specified for *Drosophila* use (anti-uNOS; PA1-039; Affinity BioReagents, *via* Cambridge BioScience, Cambridge, UK). This antibody is directed against an epitope that is closely conserved in mammalian, insect and even crustacean NOS peptides (Table 2).

This antibody has been used previously in *Drosophila* (Broderick et al., 2003; Dow and Davies, 2001; Gibbs and Truman, 1998). Specificity of this antibody has been demonstrated by immunoblotting DNOS (*Drosophila* nitric oxide synthase) protein expression (~150 kDa protein) in tubules from *dNOS* transgenic lines; overexpression of DNOS results in increased protein by western analysis, which correlates with increased NOS enzyme activity by direct assays. Also, this antibody has successfully been used for immunocytochemistry in both wild-type and *dNOS* transgenic

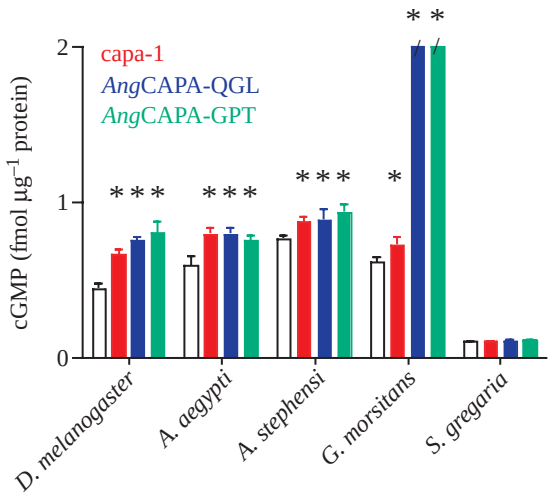

Fig. 4. cGMP levels are stimulated by capa peptides in Diptera. Basal and capa-stimulated cGMP levels in tubules from several species were measured by radioimmunoassay (RIA). Tubules were stimulated with capa-1 (red), *Ang*CAPA-QGL (blue) and *Ang*CAPA-GPT (green) peptides (10^{-7} mol l^{-1}) for 10 min. Data for each sample were calculated as fmol cGMP μg^{-1} protein (± S.E.M.; N=4–6) in order to normalise the data across species. Protein estimations were conducted by the Bradford assay. In order to aid comparison with other insects, values for *G. morsitans* tubules have been under-represented on the graph: levels of cGMP in *G. morsitans* tubules stimulated by *A. gambiae* capa peptides were 10±0.2 fmol μg^{-1} protein (*Ang*CAPA-QGL) and 9.8±0.2 fmol μg^{-1} protein (*Ang*CAPA-GPT) compared with 0.610±0.025 fmol μg^{-1} protein for control tubules. No increase in cGMP content was observed upon stimulation of *S. gregaria* tubules with either capa-1, *Ang*CAPA-QGL or *Ang*CAPA-GPT. *Statistically significant data compared with untreated tubules, where P<0.05 (Student's t-test, unpaired samples).

Drosophila tubules and in eye tissue (Broderick et al., 2003; Dow and Davies, 2001; Gibbs and Truman, 1998). Staining was visualised using a fluorescein-labelled goat anti-rabbit antibody (Diagnostics Scotland, Edinburgh, UK), used at 1:250 dilution. In order to visualise principal cell nuclei, tubules were counterstained in 1 µg ml^{-1} 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI; Sigma Aldrich, Gillingham, UK) for 2.5 min (Broderick et al., 2004). Stained tubules were mounted in VectaShield (Vector Labs, Peterborough, UK). Staining in whole-mount tubules was detected by immunofluorescence using an Axiocam imaging system (Zeiss, Welwyn Garden City, UK).

NADPH diaphorase assay for NOS activity

An assay for NOS-associated NADPH diaphorase activity in *Drosophila* tubule extracts (Broderick et al., 2003; Kean et al., 2002) was modified for analysis in 96-well plates. Intact tubules were dissected from animals (lines as described in legend to Fig. 3). For each species, either six tubules (*Drosophila*), five tubules (*Aedes* and *Anopheles*) or two tubules (*Glossina* and *Schistocerca*) were used. For each sample, tubules were placed in 93 µl of 50 mmol l^{-1} Tris HCl, pH 7.4, 1% Triton X100 and 5 µl of 10 mmol l^{-1} XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], sodium salt in 96-well plates. Samples were incubated at 25°C for 20 min. For peptide stimulations, either 1 µl of each peptide (*D. melanogaster* capa-1, *Ang*CAPA-QGL or *Ang*CAPA-GPT to a final concentration of 10^{-7} mol l^{-1}) or 1 µl phosphate-buffered saline (PBS; control) were added for a further 10 min. To each sample, either 1 µl of 100 mmol l^{-1} NADPH or 1 µl PBS (for no-substrate controls) was added, and samples were incubated at 25°C for 7 min. For each species, replicate samples were prepared under the following conditions: tubules only, tubules + NADPH, tubules + peptide, tubules + peptide + NADPH. Samples were homogenised and colorimetric analysis performed for all samples by spectrophotometry at 450 nm (Berthold Mithras plate reader; Berthold Technologies, Redbourn, UK). Blanks were prepared from incubation buffer. Blank samples, 'tubules only' samples and 'tubules + peptide' samples gave very similar readings. The overall mean of readings for blanks, 'tubules only' samples and 'tubules + peptide' samples within each experiment was subtracted from results of 'tubules + NADPH' and 'tubules + peptide + NADPH', respectively. In order to normalise data for all species, results were expressed as % increase over unstimulated tubules (± S.E.M.; N=4–6); i.e. (corrected values for 'tubules + peptide + NADPH' minus mean of corrected values for 'tubules + NADPH'/mean of corrected values for 'tubules +NADPH')×100%.

To further verify that stimulated NADPH diaphorase activity was due to NOS activation, we utilised the NOS inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME; Calbiochem, Beeston, UK) in the assays above. For these samples, L-NAME was added to samples prepared as described above, at a concentration of 2 µmol l^{-1} for 20 min prior to stimulation with capa peptides. Results were expressed as % change (± S.E.M.; N=4) of both peptide-stimulated samples and capa + L-NAME samples compared with controls (samples without either capa or L-NAME).

Tubule cGMP assays

Cyclic GMP levels were measured in pooled samples of tubules dissected from insects, as detailed in Fig. 4 legend, by radioimmunoassay (Amersham Biotrak Amerlex M; Amersham Biosciences, Chalfont St Giles, UK), as previously described (Dow et al., 1994b). Tubules were pre-incubated with the cGMP-specific phosphodiesterase inhibitor Zaprinast (Calbiochem) at 10^{-5} mol l^{-1} for 10 min. For peptide stimulations, either 1 µl of each peptide (*D. melanogaster* capa-1, *Ang*CAPA-QGL or *Ang*CAPA-GPT to a final concentration of 10^{-7} mol l^{-1}) or 1 µl PBS (control) were added for a further 10 min. Incubations were terminated with ice-cold ethanol and homogenised. The ethanol was evaporated and samples were resuspended in 0.05 mol l^{-1} sodium acetate buffer (Amersham Biosciences) and processed for cGMP content according to manufacturer's protocol. Data were normalised across insect species by expressing results as fmol cGMP μg^{-1} protein (± S.E.M.; N=4–6). Protein concentrations were determined by Bradford assay.

Fluid secretion assays

Tubule secretion was measured according to standard procedures. Intact Malpighian tubules were isolated into 9 µl drops of a freshly prepared mixture of Schneider's medium (Gibco BRL, Invitrogen Ltd) and *Drosophila* saline (1:1, v/v) under liquid paraffin, and fluid secretion rates measured in tubules as detailed elsewhere (Dow et al., 1994a). Briefly, one end of the tubule was wrapped around a metal pin and the rest of the tubule bathed on the saline drop. A nick was made near the ureter, a drop of secreted fluid collected every 10 min, and the diameter measured using an eyepiece micrometer. The volume of each droplet was calculated as $4/3\pi r^3$, where r is the radius of the droplet, and secretion rates plotted against time. Secretion was measured under basal conditions to establish a steady rate of secretion prior to stimulation with peptide(s).

For other insect species, procedures were as for *Drosophila*, with appropriate modifications to accommodate the widely differing sizes of the tubules. However, apart from *Drosophila*, all insect tubules were left in the saline bubble in the paraffin dish for at least 15 min after dissection, then wound around the pin and left for another 15 min. Tubules were then nicked to allow bubbles to form; experimental readings commenced 10 min after this. Basal rates were measured for 30 min prior to stimulation with peptides (*D. melanogaster* capa-1, *Ang*CAPA-QGL or *Ang*CAPA-GPT).

Results

Distribution of capa and its cognate receptor in insects

Although the diuretic nature of capa signalling has been well established in *Drosophila*, its phylogenetic scope has not been explored. Table 1 illustrates the sequences of the capa members identified from the lepidopteran *Manduca sexta* (Huesmann et al., 1995) and the dipterans *D. melanogaster* (Kean et al., 2002) and *A. gambiae* (present study; Riehle et al., 2002). All the peptides share the kinin-like Lx(A/P)FPRVamide motif (Kean et al., 2002), which may confer specificity of action. At present, there are no close matches for capa-like peptides in sequence data for non-insect organisms.

A gene encoding a *Drosophila* capa receptor has been identified and functionally characterised (Iversen et al., 2002; Park et al., 2002). There is a single clear homologue for the CAPA-R in the published *Anopheles* genome (BlastP; $P=5\times10^{-90}$). This is encoded by a gene with GenBank no. XP_312952. At present, there are no compelling matches in available *Aedes* or other insect sequences; the nearest match in the *Aedes* genome has been annotated as a 5-HT7 receptor. Nonetheless, if a CAPA-R homologue were found to be expressed in the tubule of another insect, it would strengthen the case for functional conservation of capa signalling. By RT-PCR with intron-spanning primers, it was possible to show that the *Anopheles* homologue was indeed expressed in the *Anopheles* tubule (Fig. 1), although the non-degenerate *Anopheles* primers did not identify a match in the *Aedes* tubule.

NOS immunoreactivity in tubules across species

Capa acts on *Drosophila* tubules to activate NOS *via* intracellular

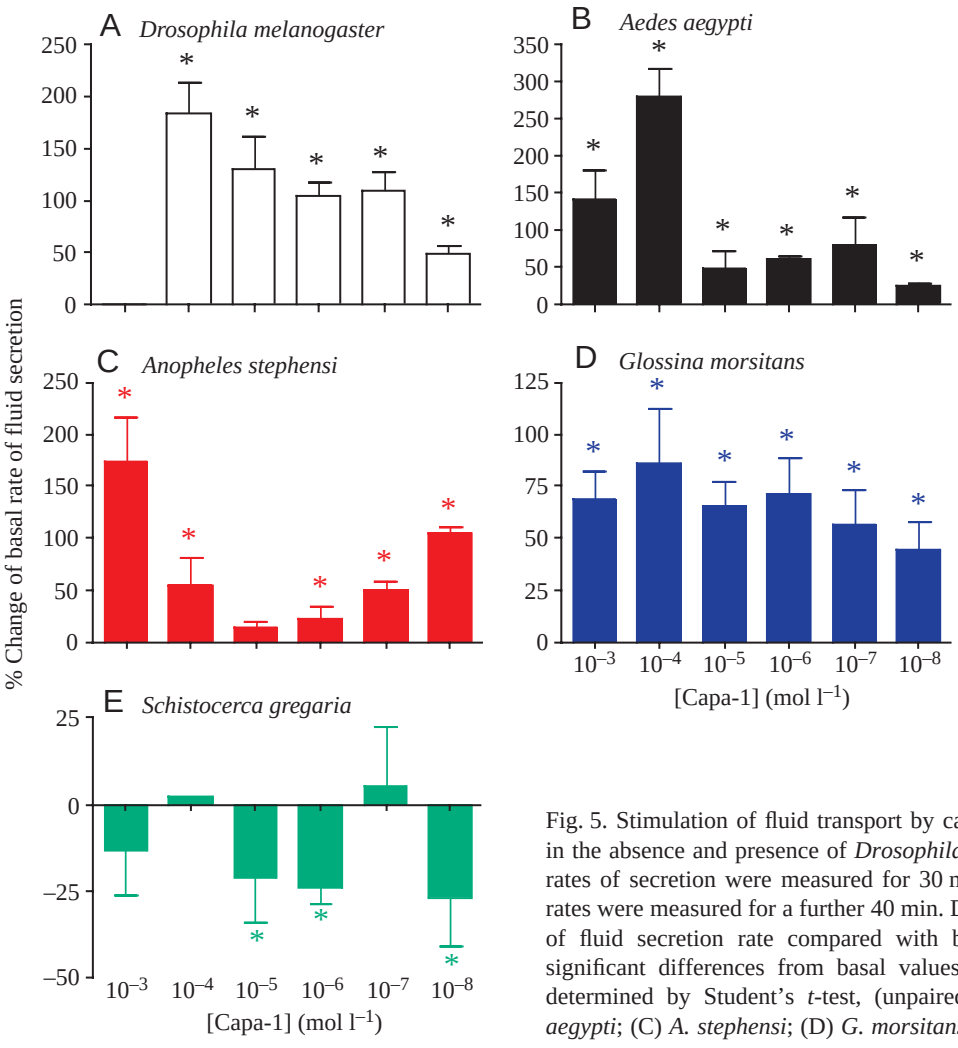

Fig. 5. Stimulation of fluid transport by capa-1. Tubule fluid secretion was measured in the absence and presence of *Drosophila* capa-1 at the concentrations shown. Basal rates of secretion were measured for 30 min prior to addition of peptides. Secretion rates were measured for a further 40 min. Data are expressed as percentage stimulation of fluid secretion rate compared with basal rate (± S.E.M.; N=6–8). Statistically significant differences from basal values are denoted by asterisks, where $P<0.05$ determined by Student's t-test, (unpaired samples). (A) *D. melanogaster*; (B) *A. aegypti*; (C) *A. stephensi*; (D) *G. morsitans* and (E) *S. gregaria*.

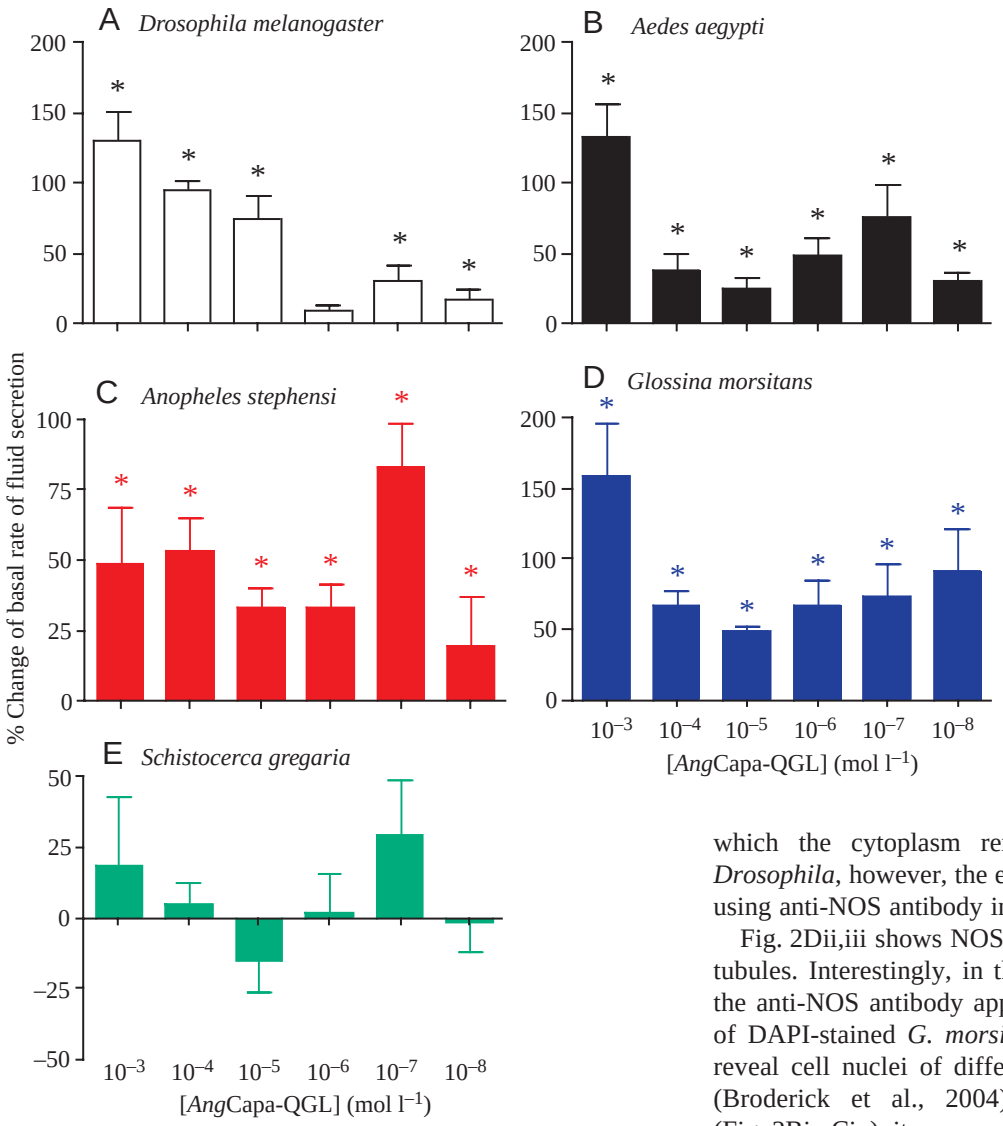

Fig. 6. Stimulation of fluid transport by *Ang*CAPA-QGL. Tubule fluid secretion was measured in the absence and presence of *Ang*CAPA-QGL at the concentrations shown. Basal rates of secretion were measured for 30 min prior to addition of peptides. Secretion rates were measured for a further 40 min. Data are expressed as percentage stimulation of fluid secretion rate compared with basal rate (± S.E.M.; N=6–8). Statistically significant differences from basal values are denoted by asterisks, where $P<0.05$ determined by Student's t-test, (unpaired samples). (A) *D. melanogaster*; (B) *A. aegypti*; (C) *A. stephensi*; (D) *G. morsitans* and (E) *S. gregaria*.

calcium: any nitridergic action of capa in other insects would thus require the presence of NOS in tubules. Accordingly, the distribution of NOS in the Malpighian tubules of other species was investigated by immunocytochemistry for NOS (Fig. 2). A universal anti-NOS antibody was utilized for these experiments; this antibody has been previously shown to be specific for *Drosophila* NOS both in the eye (Gibbs and Truman, 1998) and in tubules by immunocytochemistry (Broderick et al., 2003; Dow and Davies, 2001) and by western blotting (Broderick et al., 2003). Previous work has shown that NOS is expressed in only principal cells of *D. melanogaster* tubules (Broderick et al., 2003; Davies, 2000). Here, we show clear NOS immunoreactivity only in the main, fluid-transporting segment of the tubule (Fig. 2Aii, region marked 'm').

In mosquito tubules, NOS immunoreactivity is observed only in the cytoplasm of principal cells (examples of unstained stellate cells marked by arrows in Fig. 2Bii,iii,Cii,iii). In both *A. aegypti* and *A. stephensi*, counterstaining of cell nuclei with DAPI shows the smaller nuclei of the stellate cells (arrows in Fig. 2Biv,Civ), as in *Drosophila* (Broderick et al., 2004), of which the cytoplasm remains unstained. In contrast to *Drosophila*, however, the entire length of the tubule is stained using anti-NOS antibody in both mosquito species.

Fig. 2Dii,iii shows NOS immunoreactivity in *G. morsitans* tubules. Interestingly, in this dipteran species, staining with the anti-NOS antibody appears in all cells. Close inspection of DAPI-stained *G. morsitans* tubules (Fig. 2Div) does not reveal cell nuclei of different sizes, as in *D. melanogaster* (Broderick et al., 2004), *A. gambiae* or *A. stephensi* (Fig. 2Biv,Civ); it appears that, unlike other Diptera, *Glossina* does not have obvious stellate cells. Furthermore, staining is also observed throughout the tubule, rather than merely in the main segment.

In the orthopteran out-group, *S. gregaria*, high background staining is observed in the control tubules (Fig. 2Ei). However, increased staining is observed with the anti-NOS antibody throughout the tubule (Fig. 2Eii) at the membrane and in the cytoplasm, suggesting that expression of NOS occurs in these tubules. This is consistent with a previous report of NAPDH diaphorase activity in orthopteran tubules (*Locusta migratoria*; M. Elphick, personal communication).

It is thus clear that all the insects studied have at least some of the machinery (NOS) to produce a nitridergic response to capa.

Capa peptides elevate NADPH diaphorase activity in dipteran tubules

NADPH diaphorase staining is an obligate correlate of NOS activity, both in vertebrates and in insects (Elphick, 1997; Davies, 2000). We have previously adapted this assay for

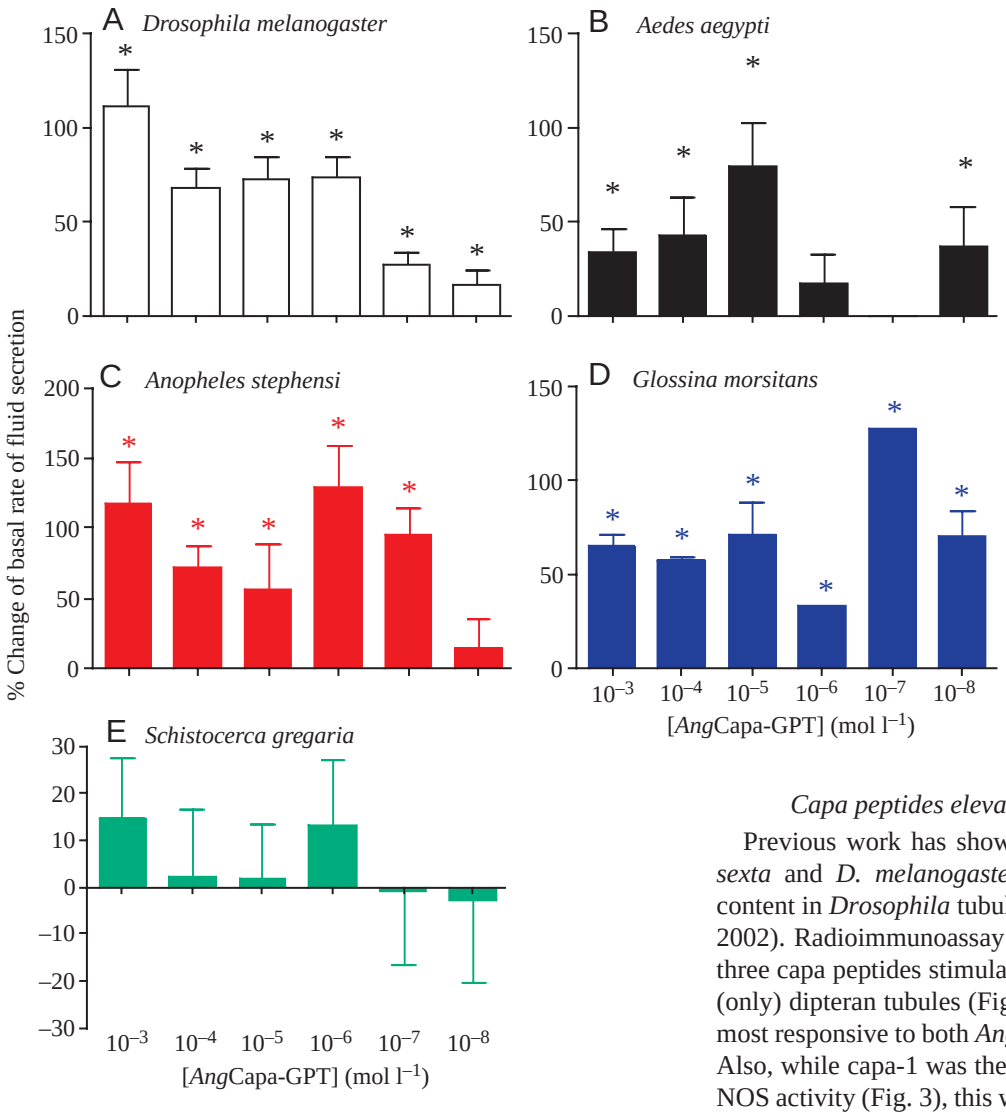

Fig. 7. Stimulation of fluid transport by *Ang*CAPA-GPT. Tubule fluid secretion was measured in the absence and presence of *Ang*CAPA-GPT at the concentrations shown. Basal rates of secretion were measured for 30 min prior to addition of peptides. Secretion rates were measured for a further 40 min. Data are expressed as percentage stimulation of fluid secretion rate compared with basal rate (± S.E.M.; N=6–8). Statistically significant differences from basal values are denoted by asterisks, where $P<0.05$ determined by Student's t-test, (unpaired samples). (A) *D. melanogaster*; (B) *A. aegypti*; (C) *A. stephensi*; (D) *G. morsitans* and (E) *S. gregaria*.

measurements *in vitro* (Kean et al., 2002), allowing quantification of NOS-associated NADPH diaphorase activity, which accurately reflects NOS activity (Broderick et al., 2003).

Results in Fig. 3A show that tubules stimulated with all capa peptides tested (i.e. *D. melanogaster* capa-1, *Ang*CAPA-QGL and *Ang*CAPA-GPT) increase NADPH diaphorase activity across the Diptera. Interestingly, capa-1 is at least as effective as, if not better than, the *A. gambiae* peptides in raising NOS activity, at least at the concentration tested, which was based on the maximum response of *D. melanogaster* tubules to capa-1 as shown in previous work (Kean et al., 2002). By contrast, although *S. gregaria* tubules both contain NOS immunoreactivity and display similar resting levels of NADPH diaphorase activity to dipteran tubules (results not shown), none of the capa peptides tested elevated NADPH diaphorase activity in this orthopteran species. In each case, L-NAME inhibited the increase in NADPH diaphorase activity to control (unstimulated) levels, confirming the association between increased NADPH diaphorase and NOS activation in these species (Elphick, 1997).

Capa peptides elevate cGMP in dipteran tubules

Previous work has shown that NO release, induced by *M. sexta* and *D. melanogaster* capa peptides, increases cGMP content in *Drosophila* tubules (Davies et al., 1995; Kean et al., 2002). Radioimmunoassay for cGMP content showed that all three capa peptides stimulated an increase in cGMP content in (only) dipteran tubules (Fig. 4). *G. morsitans* tubules were the most responsive to both *Ang*CAPA-QGL and *Ang*CAPA-GPT. Also, while capa-1 was the most effective at increasing tubule NOS activity (Fig. 3), this was not the case for the cGMP assay (Fig. 4). Finally, capa peptides do not increase cGMP in *S. gregaria* tubules [data in fmol cGMP μg^{-1} protein (± S.E.M.; N=4): unstimulated tubules: 0.097±0.003; capa-1 stimulated tubules, 0.093±0.008; *Ang*CAPA-QGL, 0.096±0.008; *Ang*CAPA-GPT, 0.106±0.003].

Activation of NO/cGMP signalling by capa peptides increases fluid secretion

Previous work has shown that fluid secretion is potently stimulated by capa-1 in *D. melanogaster* (Kean et al., 2002); Fig. 5 shows such stimulation of fluid transport by *D. melanogaster* tubules with capa-1 at an EC_{50} value of between 10^{-7} and 10^{-8} mol l^{-1}. Capa-1 also stimulates fluid transport by *A. aegypti*, *A. stephensi* and *G. morsitans* tubules. However, still higher rates of secretion occur at very high concentrations of peptide, between 10^{-3} (*A. stephensi*) and 10^{-4} mol l^{-1} (*A. aegypti*). Furthermore, *G. morsitans* tubules are only stimulated to 50% over basal levels at all concentrations of capa-1. In *S. gregaria*, capa-1 has either no significant effect on secretion or is inhibitory (10^{-5}, 10^{-6}, 10^{-8} mol l^{-1}). Similarly, capa-1 does not stimulate fluid secretion by tubules

from the dictyopteran roach *Periplaneta americana* (data not shown). Thus, of the species sampled to date, the stimulatory effects of capa-1 on tubules are confined to the Diptera.

Figs 6, 7 show the first demonstration of the physiological effects of *A. gambiae* capa peptides on tubule fluid secretion in both mosquito and other Diptera. *Drosophila* tubule secretion is stimulated in a dose-dependent manner in response to *Ang*CAPA-QGL, with an apparent EC_{50} of 10^{-5} mol l^{-1} (Fig. 6). All other dipteran tubules tested also respond to *Ang*CAPA-QGL and are more sensitive to the peptide compared with *Drosophila* tubules, especially at low concentrations [10^{-6}, 10^{-7}, 10^{-8} mol l^{-1} (*G. morsitans*)]. Apart from at 10^{-3} mol l^{-1}, tubules from *A. aegypti* and *A. stephensi* show similar responses at all concentrations tested. Also, *G. morsitans* tubules show a similar pattern of response to both mosquito species. At 10^{-7} mol l^{-1} *Ang*CAPA-QGL, stimulation of secretion rates in *A. aegypti*, *A. stephensi* and *G. morsitans* is identical. By contrast, tubule secretion rates in *S. gregaria* tubules are not significantly altered at any concentration of *Ang*CAPA-QGL.

Similarly to capa-1 and *Ang*CAPA-QGL, *Drosophila* tubules respond to all concentrations of *Ang*CAPA-GPT tested, although are most responsive at concentrations of $\geqslant 10^{-5}$ mol l^{-1}, with the maximal response occurring at 10^{-3} mol l^{-1} (Fig. 7). However, responses of all other dipteran tubules tested are similar at 10^{-5} and 10^{-6} mol l^{-1}. Maximal response of *A. aegypti* tubules occurs at 10^{-5} mol l^{-1}, of *A. stephensi* tubules at 10^{-6} mol l^{-1} and of *G. morsitans* tubules at 10^{-7} mol l^{-1} *Ang*CAPA-GPT. Interestingly, no stimulation of secretion was observed with *A. aegypti* tubules at 10^{-7} mol l^{-1}. Note also that the secretion response of these tubules to 10^{-6} mol l^{-1} *Ang*CAPA-GPT is very low; these results are reproducible (N>30). As with the other capa peptides, no significant response is obtained from *S. gregaria* tubules.

Discussion

This paper extends our detailed understanding of the unique nitridergic capa pathway from the genetic model *Drosophila* to four further species of insect in two widely spaced orders, making it one of the most wide-ranging experimental surveys of insect endocrinology to date.

In this work, we show that NOS immunoreactivity is observed in principal cells throughout *A. aegypti, A. stephensi* and *G. morsitans* tubules. By contrast, immunoreactivity for NOS is observed in all tubule cells in *S. gregaria*. For these experiments, an anti-NOS antibody to an epitope contained in all insect NOS sequences known to date was used (Table 2). Although we cannot assert that this antibody is specific to NOS alone, it faithfully reports increased NOS expression *via* an inducible NOS transgene in *Drosophila* tubules (Broderick et al., 2003) and is consistent with other measures of NOS activity reported here.

We also demonstrate that *D. melanogaster* and *A. gambiae* capa peptides all stimulate NOS activity, increase cGMP production and elicit an increase in fluid secretion rates in several dipteran species. Thus, this suggests that not only are conserved features of the capa peptide sequences functionally important but that conservation of the sequence and function of the capa receptors must also exist within the Diptera. In particular, we have identified a likely *Anopheles* homologue of the *Drosophila* CAPA-R, which is abundantly expressed in *Anopheles* tubule. Importantly, none of the capa peptides tested activate NO/cGMP signalling or elevate fluid secretion in *S. gregaria*. Indeed, capa-1 may be anti-diuretic at some concentrations (Fig. 5), although this is not linked to an increase in cGMP content (Fig. 4). The data are supported by work on *L. migratoria*, which shows that *M. sexta* CAP_{2b} does not affect fluid secretion by these tubules (Coast, 2001b; see Wegener et al., 2002). We have thus demonstrated, for the first time, physiological roles for *A. gambiae* capa peptides and that capa-stimulated fluid secretion is confined to a range of dipteran insects. We have also measured neuropeptide-stimulated secretion rates in *G. morsitans* tubules for the first time. Measurement of fluid secretion in the tsetse fly was first published nearly 30 years ago (Gee, 1976a,b). More recent work has re-visited cAMP-stimulated fluid secretion by *G. morsitans* tubules (Isaacson and Nicolson, 1994). However, our recent development of *Glossina* tubule physiology will allow study of a critical tissue in a disease vector. The demonstration of conservation of capa signalling in medically important insect vectors suggests new possibilities for novel insecticide targets for pest control.

Importantly, we extend the phylogenetic scope of diuretic cGMP signalling beyond *Drosophila*. It is apparent that cGMP can act as an anti-diuretic signal in some insects. For example, in *T. molitor*, two anti-diuretic hormones that act *via* cGMP have been isolated (Eigenheer et al., 2002, 2003). However, the existence of anti-diuretic, cGMP-mobilising hormones in some insects need not point to a universal mode of action by cGMP in insect tubules. Rather, this suggests a critical distinction in the use of cGMP by different animals and, more than that, a relevant role of cell or tissue concentration of cGMP in physiology.

Locust tubules contain NOS but do not respond to capa. This result does not, however, rule out nitridergic signalling in non-dipteran tubules. NOS-encoding genes have been characterized from multiple orders of insect (Davies, 2000), and all contain well-conserved calmodulin-binding domains, implying that, like *Drosophila* NOS, they are calcium/calmodulin regulated. It is thus probable that any neuropeptide that elevates calcium in *Schistocerca* (or indeed any insect) tubule will activate NOS to generate NO. The capa peptides perform such a role in Diptera, but our evidence suggests that they do not in Orthoptera. Consistent with this argument, calcium has been shown to be important in *L. migratoria* tubule stimulation by a partially purified hormone, and cGMP has been shown to be diuretic (Morgan and Mordue, 1985). Of course, the generation of NO in a tissue does not imply that it will be sensed by soluble guanylate cyclase in the same tissue. In the future, it will be of interest to follow the phylogenetic distribution of NO-sensing in insect tubules, in particular those from non-dipteran species, including orthopteran insects.

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References

Beyenbach, K. (2003). Transport mechanisms of diuresis in Malpighian tubules of insects. *J. Exp. Biol.* **206**, 3845-3856.

Broderick, K. E., MacPherson, M. R., Regulski, M., Tully, T., Dow, J. A. T. and Davies, S. A. (2003). Interactions between epithelial nitric oxide signaling and phosphodiesterase activity in *Drosophila*. *Am. J. Physiol. Cell Physiol.* **285**, C1207-C1218.

Broderick, K. E., Kean, L., Dow, J. A. T., Pyne, N. J. and Davies, S. A. (2004). Ectopic expression of bovine type 5 phosphodiesterase confers a renal phenotype in *Drosophila*. *J. Biol. Chem.* **279**, 8159-8168.

Coast, G. M. (2001a). Diuresis in the housefly (*Musca domestica*) and its control by neuropeptides. *Peptides* **22**, 153-160.

Coast, G. M. (2001b). The neuroendocrine regulation of salt and water balance in insects. *Zoology* **103**, 179-188.

Davies, S.-A. (2000). Nitric oxide signalling in insects. *Insect Biochem. Mol. Biol* **30**, 1123-1138.

Davies, S. A., Huesmann, G. R., Maddrell, S. H. P., O'Donnell, M. J., Skaer, N. J. V., Dow, J. A. T. and Tublitz, N. J. (1995). CAP_{2b}, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion *via* cGMP. *Am. J. Physiol.* **269**, R1321-R1326.

Davies, S. A., Stewart, E. J., Huesmann, G. R., Skaer, N. J. V., Maddrell, S. H. P., Tublitz, N. J. and Dow, J. A. T. (1997). Neuropeptide stimulation of the nitric oxide signaling pathway in *Drosophila melanogaster* Malpighian tubules. *Am. J. Physiol.* **42**, R823-R827.

Dimopoulos, G., Seeley, D., Wolf, A. and Kafatos, F. C. (1998). Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *EMBO J.* **17**, 6115-6123.

Dow, J. A. T. (1999). The multifunctional *Drosophila melanogaster* V-ATPase is encoded by a multigene family. *J. Bioenerg. Biomembr.* **31**, 75-83.

Dow, J. A. T. and Davies, S. A. (2001). The *Drosophila melanogaster* Malpighian tubule. *Adv. Insect Physiol.* **28**, 1-83.

Dow, J. A. T. and Davies, S. A. (2003). Integrative physiology, functional genomics and epithelial function in a genetic model organism. *Physiol. Rev.* **83**, 687-729.

Dow, J. A. T., Maddrell, S. H., Gortz, A., Skaer, N. J., Brogan, S. and Kaiser, K. (1994a). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* **197**, 421-428.

Dow, J. A. T., Maddrell, S. H., Davies, S. A., Skaer, N. J. and Kaiser, K. (1994b). A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*. *Am. J. Physiol.* **266**, R1716-R1719.

Eigenheer, R. A., Nicolson, S. W., Schegg, K. M., Hull, J. J. and Schooley, D. A. (2002). Identification of a potent antidiuretic factor acting on beetle Malpighian tubules. *Proc. Natl. Acad. Sci. USA* **99**, 84-89.

Eigenheer, R. A., Wiehart, U. M., Nicolson, S. W., Schoofs, L., Schegg, K. M., Hull, J. J. and Schooley, D. A. (2003). Isolation, identification and localization of a second beetle antidiuretic peptide. *Peptides* **24**, 27-34.

Elphick, M. R. (1997). Localization of nitric oxide synthase using NADPH diaphorase histochemistry. *Methods Mol. Biol.* **72**, 153-158.

Gee, J. D. (1976a). Active transport of sodium by the Malpighian tubules of the tsetse fly *Glossina morsitans*. *J. Exp. Biol.* **64**, 357-368.

Gee, J. D. (1976b). Fluid secretion by the Malpighian tubules of the tsetse fly *Glossina morsitans*: the effects of ouabain, ethacrynic acid and amiloride. *J. Exp. Biol.* **65**, 323-332.

Gibbs, S. M. and Truman, J. W. (1998). Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe in *Drosophila*. *Neuron* **20**, 83-93.

Huesmann, G. R., Chung, C. C., Loi, P. K., Lee, T. D., Swiderek, K. and Tublitz, N. J. (1995). Amino acid sequence of CAP2b, an insect cardioacceleratory peptide from the tobacco hornworm *Manduca sexta*. *FEBS Lett.* **371**, 311-314.

Isaacson, L. and Nicolson, S. (1994). Concealed transepithelial potentials and current rectification in tsetse fly Malpighian tubules. *J. Exp. Biol.* **186**, 199-213.

Iversen, A., Cazzamali, G., Williamson, M., Hauser, F. and Grimmelikhuijzen, C. J. (2002). Molecular cloning and functional expression of a Drosophila receptor for the neuropeptides capa-1 and –2. *Biochem. Biophys. Res. Commun.* **299**, 628-633.

Kay, I., Patel, M., Coast, G. M., Totty, N. F., Mallet, A. I. and Goldsworthy, G. J. (1992). Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana L. Regul. Pept.* **42**, 111-122.

Kean, L., Cazenave, W., Costes, L., Broderick, K. E., Graham, S., Pollock, V. P., Davies, S. A., Veenstra, J. A. and Dow, J. A. T. (2002). Two nitridergic peptides are encoded by the gene capability in Drosophila melanogaster. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R1297-R1307.

Laenen, B., Verhaert, P., Schoofs, L., Steels, P. and Van Kerkhove, E. (1999). Partial identification of a peptide that stimulates the primary urine production of single isolated Malpighian tubules of the forest ant, *Formica polyctena*. *J. Insect Physiol.* **45**, 743-753.

Laenen, B., De Decker, N., Steels, P., Van Kerkhove, E. and Nicolson, S. (2001). An antidiuretic factor in the forest ant: purification and physiological effects on the Malpighian tubules. *J. Insect Physiol.* **47**, 185-193.

Luckhart, S., Vodovotz, Y., Cui, L. and Rosenberg, R. (1998). The mosquito *Anopheles stephensi* limits malaria. parasite development with inducible synthesis of nitric oxide. *Proc. Natl. Acad. Sci. USA* **95**, 5700-5705.

MacPherson, M. R., Pollock, V. P., Broderick, K. B., Kean, L., O'Connell, F. C., Dow, J. A. T. and Davies, S.-A. (2001). Model organisms: new insights into ion channel and transporter function: L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster*. *Am. J. Physiol. Cell Physiol.* **280**, C394-C407.

Morgan, P. J. and Mordue, W. (1985). The role of calcium in diuretic hormone action on locust Malpighian tubules. *Mol. Cell. Endocrinol.* **40**, 221-231.

O'Donnell, M. J. and Spring, J. H. (2000). Modes of control of insect Malpighian tubules: synergism, antagonism, cooperation and autonomous regulation. *J. Insect Physiol.* **46**, 107-117.

Park, Y., Kim, Y. J. and Adams, M. E. (2002). Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc. Natl. Acad. Sci. USA* **99**, 11423-11428.

Pullikuth, A., Filippov, V. and Gill, S. (2003). Phylogeny and cloning of ion transporters in mosquitoes. *J. Exp. Biol.* **206**, 3857-3868.

Quinlan, M. C., Tublitz, N. J. and O'Donnell, M. J. (1997). Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stal: The peptide CAP(2b) and cyclic GMP inhibit Malpighian tubule fluid secretion. *J. Exp. Biol.* **200**, 2363-2367.

Riegel, J. A., Maddrell, S. H., Farndale, R. W. and Caldwell, F. M. (1998). Stimulation of fluid secretion of malpighian tubules of drosophila melanogaster meig. by cyclic nucleotides of inosine, cytidine, thymidine and uridine. *J. Exp. Biol.* **201**, 3411-3418.

Riehle, M. A., Garcynski, S. F., Crim, J. W., Hill, C. A. and Brown, M. R. (2002). Neropeptides and peptide hormones in *Anopheles gambiae*. *Science* **298**, 172-175.

Rosay, P., Davies, S. A., Yu, Y., Sozen, A., Kaiser, K. and Dow, J. A. T. (1997). Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci.* **110**, 1683-1692.

Schoofs, L., Veelaert, D., Vanden Broeck, J. and De Loof, A. (1997). Peptides in the locusts, *Locusta migratoria* and *Schistocerca gregaria*. *Peptides* **18**, 145-156.

Spring, J. H. and Clark, T. M. (1990). Diuretic and antidiuretic factors which act on the Malpighian tubules of the house cricket, *Acheta domesticus*. *Prog. Clin. Biol. Res.* **342**, 559-564.

Te Brugge, V. A., Schooley, D. A. and Orchard, I. (2002). The biological activity of diuretic factors in *Rhodnius prolixus*. *Peptides* **23**, 671-681.

Wegener, C., Herbert, Z., Eckert, M. and Predel, R. (2002). The periviscerokinin (PVK) peptide family in insects: evidence for the inclusion of CAP(2b) as a PVK family member. *Peptides* **23**, 605-611.

Wiehart, U. I., Nicolson, S. W., Eigenheer, R. A. and Schooley, D. A. (2002). Antagonistic control of fluid secretion by the Malpighian tubules of *Tenebrio molitor*: effects of diuretic and antidiuretic peptides and their second messengers. *J. Exp. Biol.* **205**, 493-501.